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Synthetic Lethality of the *lytE cwlO* Genotype in *Bacillus subtilis* Is Caused by Lack of D,L-Endopeptidase Activity at the Lateral Cell Wall

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Bacterial peptidoglycan acts as an exoskeleton to protect the bacterial cell. Although peptidoglycan biosynthesis by penicillin-binding proteins is well studied, few studies have described peptidoglycan disassembly, which is necessary for a dynamic structure that allows cell growth. In *Bacillus subtilis*, more than 35 genes encoding cell wall lytic enzymes have been identified; however, only two D,L-endopeptidases (*lytE* and *cwlO*) are involved in cell proliferation. In this study, we demonstrated that the D,L-endopeptidase activity at the lateral cell wall is essential for cell proliferation. Inactivation of LytE and CwlO by point mutation of the catalytic residues caused cell growth defects. However, the forced expression of LytF or CwlS, which are paralogs of LytE, did not suppress *lytE cwlO* synthetic lethality. Subcellular localization studies of these D,L-endopeptidases showed LytF and CwlS at the septa and poles, CwlO at the cylindrical part of the cell, and LytE at the septa and poles as well as the cylindrical part. Furthermore, construction of N-terminal and C-terminal domain-swapped enzymes of LytE, LytF, CwlS, and CwlO revealed that localization was dependent on the N-terminal domains. Only the chimeric proteins that were enzymatically active and localized to the sidewall were able to suppress the synthetic lethality, suggesting that the lack of D,L-endopeptidase activity at the cylindrical part of the cell leads to a growth defect. The functions of LytE and CwlO in cell morphogenesis were discussed.

Autolysins are bacterial cell wall lytic enzymes found in all bacteria that possess peptidoglycan. In the *Bacillus subtilis* genome, more than 35 definite or probable autolysin genes have been identified and shown to be involved in cell morphogenesis, cannibalism, sporulation, and germination (22, 25). The bacterial peptidoglycan sacculus requires a dynamic structure for cell elongation and separation; therefore, a balance between peptidoglycan synthesis and disassembly is essential for cell proliferation. Although a number of autolysins are thought to be involved in peptidoglycan disassembly, none have been found to be essential for cell growth, perhaps due to their functional redundancy. However, it was recently reported that disruption of both *lytE* and *cwlO* in *B. subtilis* is lethal (4). To date, this is the sole report of an autolysin mutant of *B. subtilis* with a serious growth defect. Bisicchia et al. (4) also demonstrated that *cwlO* depletion in a *lytE*-disrupted background strain impairs cell elongation.

LytE and CwlO are D,L-endopeptidases that hydrolyze the linkage of D-γ-glutamyl-*meso*-diaminopimelic acid in peptidoglycan (13, 27). The *B. subtilis* genome contains seven D,L-endopeptidase genes. The mature forms of LytE, LytF, and CwlS all contain N-terminal LysM repeats, although the number of LysM domains differs, and C-terminal D,L-endopeptidase domains belonging to the NlpC/P60 family. Although phenotypes of single-gene knockout mutants were indistinguishable from that of the wild type, multiple gene disruptions led to a chained-cell morphology (10, 13, 19), suggesting that these proteins are involved in cell separation. In contrast, CwlO contains a domain with unknown function at the N terminus and a D,L-endopeptidase domain at the C terminus. The phenotype of the *cwlO* mutant was also indistinguishable from that of the wild type, but the *lytE cwlO* double disruption leads to synthetic lethality (4, 27). Two D,L-endopeptidase genes (*pgdS* and *cwlT*) are not likely to be involved in cell morphology, because the *pgdS* gene encodes a poly-γ-glutamic acid degradase, and the *cwlT* gene is part of an integrative and conjugative element (11, 23). The other gene is a function-unknown *ykfC*. Results of these previous studies indicate that LytE, LytF, and CwlS are cell separation enzymes, and LytE and CwlO are associated with cell growth. Thus, although their catalytic domains show high amino acid sequence similarity, these enzymes play different physiological roles in cell morphology. To elucidate the roles of LytE and CwlO in cell morphogenesis, we investigated the main factors causing synthetic lethality in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains, plasmids, and primers used in this study are listed in Table 1 and Tables S1 and S2 in the supplemental material, respectively. *B. subtilis* 168 was used as the parent strain throughout this study. The details of the strains and plasmids constructs used in this study are presented in the supplemental material. All constructed strains were confirmed by PCR.

General methods. The *B. subtilis* and *Escherichia coli* strains were grown at 37°C in Luria broth (LB) (21). When required, antibiotics and chemical inducers were added in the following concentrations: ampicillin, 100 μg/ml; tetracycline, 5 μg/ml; kanamycin, 25 μg/ml; spectinomycin, 50 μg/ml; erythromycin, 0.3 μg/ml chloramphenicol, 5 μg/ml; and IPTG (isopropyl β-D-1-thiogalactopyranoside), 1 mM; and xylose, 1%.

DNA manipulation and *E. coli* transformation were performed using standard methods (21). *B. subtilis* transformation was performed by conventional transformation procedures (1).

Sample preparation for IFM. Cells harvested from an overnight culture in LB medium were diluted 50-fold in 5 ml of fresh LB medium. The cells were grown to the late exponential growth phase (optical density at 600 nm [OD_{600}] = 2.0), and then the precultured cells were inoculated into fresh LB medium to give an initial absorbance equivalent to an OD_{600}

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TABLE 1 Bacterial strains used in this study

Strain	Relevant genotype	Source or reference[a]
E. coli strains		
JM109	*recA1 endA1 gyrA96 thi-1 hsdR17 relA1 supE44* Δ(*lac-proAB*)/F′ [*traD36 proAB lacI*^{q}*lacZ*ΔM15]	Takara
C600	*supE44 hsdR17 thi-1 thr-1 leuB6 lacY1 tonA21*	Laboratory stock
M15/pREP4	*lac ara gal mtl* F$^-$ *recA*$^+$ *uvr*$^+$*/lacI* Kan	Qiagen
B. subtilis strains		
168	*trpC2*	S. D. Ehrlich
FTD	*trpC2 lytE*::*tet*	30
OH001	*trpC2 cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	pXyl-cwlO → 168
OH002	*trpC2 lytE*::*tet cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	OH001 → 168FTD
OH003	*trpC2 lytE*::pM4LYTE	pM4LYTE → 168
OH004	*trpC2 lytE*::*lytE*-6×FLAG *cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	pCA6FLCF → OH001
OH005	*trpC2 lytE*::*lytE*(*C247S*)-6×FLAG *cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	$pCALE_{C247S}$ → OH001
OH006	*trpC2 cwlO*::*cwlO*-6×FLAG *lytE*::pM4LYTE (P_{spac}-*lytE*)	Supplemental material
OH007	*trpC2 cwlO*::*cwlO*(*C377S*)-6×FLAG *lytE*::pM4LYTE (P_{spac}-*lytE*)	Supplemental material
OH008	*trpC2 lytF*::pM4LYTF	pM4LYTF → 168
OH009	*trpC2 lytE*::*tet cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*) *lytF*::pM4LYTF (P_{spac}-*lytF*)	OH008 → OH002
BKD	*trpC2 lytC*::Kan	27
OH010	*trpC2 lytE*::*tet cwlO*::pXyl-cwlO *lytF*::pM4LYTF *lytC*::Kan	168BKD → OH009
OH011	*trpC2 cwlS*::pM4SDΔojL	pM4SDΔojL → 168
OH012	*trpC2 lytE*::*tet cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*) *cwlS*::pM4SDΔojL (P_{spac}-*cwlS*)	OH011 → OH002
WEC	*trpC2* Δ*wprA* Δ*epr*	30
WECLytF6FL[b]	*trpC2* Δ*wprA* Δ*epr lytF*::pCA6FLCE	30
WECLytE6FL[b]	*trpC2* Δ*wprA* Δ*epr lytE*::pCA6FLCF	30
WECS6FL	*trpC2* Δ*wprA* Δ*epr cwlS*::pCA6FLCS	30
WECO6FL	*trpC2* Δ*wprA* Δ*epr cwlO*::pCA6FLCO	pCA6FLCO → WEC
OH013	*trpC2* Δ*wprA* Δ*epr*/pDG-O6FL	pDGO6FL → WEC
OH014	*trpC2* Δ*wprA* Δ*epr lytF*::pCA6FLCWB_E	pCA6FLCWB_E → WEC
OH015	*trpC2* Δ*wprA* Δ*epr lytE*::pCA6FLCWB_F	pCA6FLCWB_F → WEC
OH016	*trpC2* Δ*wprA* Δ*epr cwlS*::pCA6FLCWB_S	pCA6FLCWB_S → WEC
OH017	*trpC2* Δ*wprA* Δ*epr cwlO*::pCA6FLNTD_O	pCA6FLNTD_O → WEC
OH018	*trpC2* Δ*wprA* Δ*epr*/pDGNO6FL	pDGNO6FL → WEC
OH019	*trpC2 lytE*::pCA-FbEcII ($N_{LytE}C_{LytF}$) *cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	pCA-FbEcII → OH002
OH020	*trpC2 lytE*::pCA-FbSc ($N_{LytE}C_{CwlS}$) *cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	pCA-FbSc → OH002
OH021	*trpC2 lytE*::pBlue-FtEbkan (5′-*lytF kan*) *cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	pBlue-FtEbkan → OH002
OH022	*trpC2 lytE*::$N_{LytF}C_{LytE}$ *cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	Supplemental material
OH023	*trpC2 cwlO*::$N_{CwlO}C_{LytF}$ *lytE*::pM4LYTE (P_{spac}-*lytE*)	Supplemental material
OH024	*trpC2 cwlO*::$N_{CwlO}C_{CwlS}$ *lytE*::pM4LYTE (P_{spac}-*lytE*)	Supplemental material

[a] Sources shown before and after the arrows indicate donor DNA and recipient cells of transformation, respectively.
[b] The previous strain names, WECE6FL and WECF6FL (30), are changed to WECLytF6FL and WECLytE6FL, respectively, to avoid the confusion of gene names.

of 0.001. Cells corresponding to 0.3 of the OD_{600} unit for WECLytE6FL (LytE-6×FLAG), OH015 (CWB_{LytE}-6×FLAG), WECO6FL (CwlO-6×FLAG), OH013 (overexpressed CwlO-6×FLAG), or OH018 (overexpressed NTD_{CwlO}-6×FLAG) were collected when each culture reached an OD_{600} of 0.1. As described below, LytE-6×FLAG and CwlO-6×FLAG were functional for *B. subtilis* cell proliferation. Likewise, cells corresponding to 0.3 of the OD_{600} unit were collected for WECLytF6FL (LytF-6×FLAG) and OH014 (CWB_{LytF}-6×FLAG) when the cultures reached an OD_{600} of 0.6. Similarly, cells corresponding to 0.3 of the OD_{600} unit were collected for WECS6FL (CwlS-6×FLAG) and OH016 (CWB_{CwlS}-6×FLAG) when each culture reached an OD_{600} of 2.0. To determine the subcellular localization of the domain-swapped chimeric enzymes, cells were collected when the cultures reached an OD_{600} of 0.3 (for chimeric proteins transcribed from the *lytE* promoter) or an OD_{600} of 0.1 (for those transcribed from the *cwlO* promoter). Cell samples were prepared for immunofluorescence microscopy (IFM) as described previously (30).

Fluorescence microscopy. Fluorescence microscopy was performed as described previously (29) with an Olympus BX61 microscope equipped with a BX-UCB control unit, a UPPlan Apo Fluorite phase-contrast objective (×100 magnification; numerical aperture, 1.3), and a standard rhodamine filter set for visualizing Cy3. Exposure times were 0.1 s for phase-contrast microscopy and 0.1 s (gain 2) for Cy3. The cells were photographed with a charge-coupled-device camera (CoolSNAP HQ; Nippon Roper) driven by MetaMorph software (version 4.6; Universal Imaging). For Cy3 imaging, out-of-focus light was removed using the two-dimensional deconvolution utility of the AutoDeblur software. All images were processed with Adobe Photoshop software.

Western blot analysis and zymography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 14% (wt/vol) polyacrylamide gels as described previously (15). For Western blot analysis, the 6×FLAG-fused proteins were separated by 14% SDS-PAGE gels. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Invitrogen) in a transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol, 0.1% SDS) by using a semidry blotting system (Bio-Rad). Immunoblot detection was carried out as described in the instruction manual for the ECL Plus Western blotting detection system (Invitrogen) using a mouse anti-FLAG M2 monoclonal antibody (Sigma) and horseradish peroxidase-labeled anti-mouse IgG antibody. Zymography was performed as described previously using 14% SDS-PAGE gels containing 0.5 mg/ml *B. subtilis* cell wall extract (17). The cell wall derived from *B. subtilis* 168 was prepared as described previously (8, 19). Renaturation was performed at 37°C in a rena-

turation solution (25 mM Tris-HCl [pH 7.2], 1% [vol/vol] Triton X-100) as described previously (10).

RESULTS

D,L-Endopeptidase activity of LytE or CwlO is essential for cell proliferation. The catalytic domains of LytE and CwlO belong to the NlpC/P60 family, which hydrolyzes the γ-D-glutamyl-*meso*-diaminopimelic acid linkage or *N*-acetylmuramoyl-L-alanine linkage. In this superfamily of papain-like enzymes, a conserved cysteine residue was predicted to be a catalytic residue on amino acid sequence alignment (2, 16). Recently, the three-dimensional structures of NlpC/P60 enzymes were reported (Spr from *E. coli*, ABA23003 from *Anabaena variabilis*, and ACC79413 from *Nostoc punctiforme*) (3, 26). In these enzymes, the conserved cysteine residues are located at a predicted active site and are structurally conserved. To determine whether the conserved cysteine residues are involved in the catalytic activity of D,L-endopeptidases, we constructed point mutations in LytE and CwlO, replacing the conserved cysteine residue with a serine residue ($LytE_{C247S}$ and $CwlO_{C377S}$). To evaluate the lytic activities of these mutated enzymes, the intact or mutated catalytic domains of LytE and CwlO were expressed in *E. coli*, and zymography was carried out with the cell lysates by using *B. subtilis* cell wall as a substrate (see Fig. S1B in the supplemental material). The intact catalytic domains of LytE and CwlO exhibited cell wall-degrading activity, but mutants in which the cysteine residue had been replaced appeared to be inactive. This finding suggests that the conserved cysteine residue is important for the catalytic activity of NlpC/P60 enzymes.

Next, we examined whether the D,L-endopeptidase activities of LytE and CwlO are involved in the synthetic lethality of the *lytE cwlO* double mutants (Fig. 1A and B). OH004 (*lytE*-6×FLAG P_{xyl}-*cwlO*) grew normally without xylose induction of CwlO, indicating that LytE-6×FLAG was functional. In contrast, the growth of OH005 [*lytE*(*C247S*)-6×FLAG P_{xyl}-*cwlO*] was normal in the presence of xylose but was arrested in the absence of xylose. Similarly, CwlO-6×FLAG was functional, but OH007 [*cwlO*(*C377S*)-6×FLAG P_{spac}-*lytE*] showed growth arrest without LytE induction by IPTG. These results indicate that the D,L-endopeptidase activity of either LytE or CwlO is essential for cell proliferation.

As described above, LytE, LytF, and CwlS exhibit similar domain structures. However, *lytE* expression is regulated by σ^A and σ^H, *cwlO* expression is regulated by σ^A, and *lytF* and *cwlS* are regulated by σ^D and σ^H, respectively (5, 13, 19, 27). The σ^D and σ^H regulons are induced later than the σ^A regulon. Therefore, although LytF and CwlS can suppress the synthetic lethality, the LytE CwlO double-depleted cells may be dead before LytF or CwlS can be expressed. Consequently, OH009 (Δ*lytE* P_{xyl}-*cwlO* P_{spac}-*lytF*) and OH012 (Δ*lytE* P_{xyl}-*cwlO* P_{spac}-*cwlS*) were constructed to determine whether induction of LytF or CwlS could suppress the synthetic lethality. These strains were cultured in the presence of 1 mM IPTG to induce LytF or CwlS and in the presence or absence of 1% xylose to induce CwlO (Fig. 1C and D). Both strains grew normally when CwlO was expressed; however, growth was arrested by CwlO depletion, even though LytF or CwlS was expressed. The hydrolytic activities of induced LytF and CwlS were confirmed by zymography with *B. subtilis* cell wall as a substrate (see Fig. S2 in the supplemental material). We found that LytF and CwlS are not able to suppress the LytE CwlO-depleted synthetic lethality, even though their domain structures are similar to that of LytE.

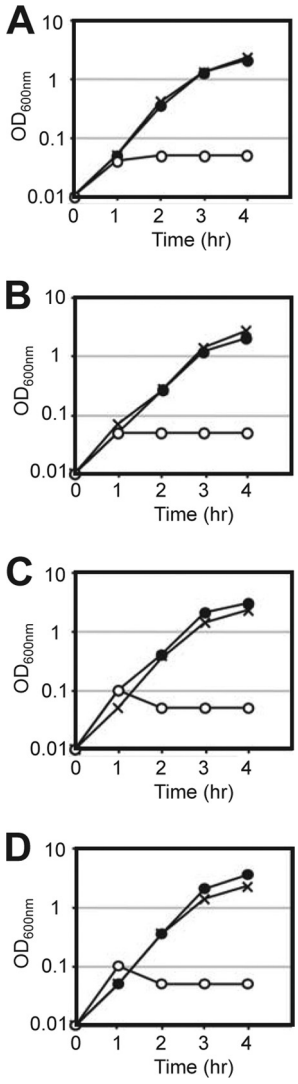

FIG 1 D,L-Endopeptidase activity of LytE and CwlO is important for cell proliferation, and LytF or CwlS induction could not suppress *lytE cwlO* synthetic lethality. Strains were precultured with the appropriate inducer until late exponential phase (OD_{600} = 2.0). An aliquot of each culture was washed and inoculated into fresh medium with or without the inducer to an OD_{600} of 0.01. The × symbol in panels A to D indicates the wild-type 168 strain. (A) Growth of OH005 [*lytE*(*C247S*)-6×FLAG P_{xyl}-*cwlO*; open circles] and OH004 (*lytE*-6×FLAG P_{xyl}-*cwlO*; closed circles). Xylose (1%) was added to the preculture, but CwlO expression was not induced by xylose in the main culture. (B) Growth of OH007 [*cwlO*(*C377S*)-6×FLAG P_{spac}-*lytE*; open circles] and OH006 (*cwlO*-6×FLAG P_{spac}-*lytE*; closed circles). IPTG (1 mM) was added to the preculture, but LytE expression was not induced by IPTG in the main culture. (C) Growth of OH009 (Δ*lytE* P_{xyl}-*cwlO* P_{spac}-*lytF*). The strain was cultured with 1 mM IPTG to induce LytF expression and with 1% xylose to induce CwlO induction (closed circles) or without xylose (open circles). (D) Growth of OH012 (Δ*lytE* P_{xyl}-*cwlO* P_{spac}-*cwlS*). The strain was cultured with 1 mM IPTG to induce CwlS expression and with 1% xylose to induce CwlO expression (closed circles) or without xylose (open circles).

Subcellular localization of *B. subtilis* D,L-endopeptidases. The C-terminal D,L-endopeptidase domains of LytE, LytF, CwlS, and CwlO show strong sequence similarity. In contrast, the N-terminal domains of LytE, LytF, and CwlS contain different numbers of the LysM repeats, and the N terminus of CwlO contains a COG3883 domain. Although the D,L-endopeptidase activ-

ity of either LytE or CwlO is essential for cell proliferation, forced expression of LytF or CwlS did not suppress the *lytE cwlO* synthetic lethality. These results suggest that the N-terminal domains are important for the function of the D,L-endopeptidases. Previously, we reported that *B. subtilis* WE1, a strain with defects in extracellular proteases WprE and Epr, accumulates D,L-endopeptidases on the cell surface (29). Therefore, we evaluated the subcellular localization of FLAG-tagged LytE, LytF, CwlS, and CwlO (full-length proteins and N-terminal domains) by IFM with *wprE epr*-deleted WEC background strains. Because these D,L-endopeptidases are regulated by different σ factors, we also evaluated the localization of these enzymes during different growth phases. Full-length LytE and CwlO and their N-terminal domains (CWB_{LytE} and NTD_{CwlO}, respectively) were observed during early exponential growth phase (OD_{600} = 0.1), full-length LytF and its N-terminal domain (CWB_{LytF}) were observed in mid-exponential growth phase (OD_{600} = 0.6), and full-length CwlS and its N-terminal domain (CWB_{CwlS}) were observed in early stationary phase (OD_{600} = 2.0). The results showed that LytE is localized at the cell septa, poles, and sidewall (Fig. 2A). LytF-6×FLAG and CwlS-6×FLAG were localized at the cell septa and poles, but neither was detected at the lateral cell wall (Fig. 2C and E). CwlO-6×FLAG expressed from the intact promoter was weakly detected at the lateral cell wall but not at the septa or poles (Fig. 2G). To better assess CwlO localization, we then used a CwlO-6×FLAG-overexpressing strain (Fig. 2H), which increased cell surface CwlO-6×FLAG expression to 2.4 times that of normal, as determined by Western blot analysis (data not shown). The overexpressed CwlO-6×FLAG was more clearly visualized at the sidewall but not detected at the cell septa or poles. To determine whether the localization of these D,L-endopeptidases depends on the N-terminal domain, we investigated the subcellular localization of the N-terminal domains under the same conditions used for the full-length proteins (Fig. 2B, D, F, and I). The localization pattern of each N-terminal domain was identical to that of the corresponding full-length protein, indicating that these D,L-endopeptidases localized on the cell surface through their N-terminal domains.

Characterization of domain-swapped D,L-endopeptidases. IFM analysis demonstrated that LytF and CwlS (involved in cell separation) localize to the septa and poles, CwlO (involved in cell elongation) localizes to the lateral cell wall, and LytE (involved both in cell separation and elongation) localizes to the septa, poles, and lateral cell wall. These results suggest that the functions of these D,L-endopeptidases depend on their subcellular localization. To test this hypothesis, we generated domain-swapped D,L-endopeptidases and examined their ability to suppress the *lytE cwlO* synthetic lethality.

Domain-swapped D,L-endopeptidases (other than $N_{LytF}C_{LytE}$) were generated by C-terminal domain substitution at the original genetic loci of the N-terminal domains. For example, $N_{LytE}C_{CwlS}$ was constructed by substituting the C-terminal domain of LytE with that of CwlS at the *lytE* locus. Thus, the chimeric genes were transcribed from the promoters of the gene encoding the N-terminal domain. However, $N_{LytF}C_{LytE}$ was constructed by substituting the N-terminal domain of LytE with that of LytF at the *lytE* locus; the chimeric gene was transcribed from the *lytE* promoter. All chimeric proteins were fused to a 6×FLAG tag at the C terminus to evaluate their expression and localization. Expression was confirmed by Western blot analysis, and the chimeric proteins were detected at positions corresponding to the predicted molecular sizes (Fig. 3A). Enzyme activity was assessed by zymography using the *B. subtilis* cell wall as a substrate (Fig. 3B). The results show that the chimeric enzymes containing the CwlO N-terminal domain did not retain cell wall-degrading activity. The C-terminal D,L-endopeptidase regions of $N_{CwlO}C_{LytF}$ and $N_{CwlO}C_{CwlS}$ are the same as those of $N_{LytE}C_{LytF}$ and $N_{LytE}C_{CwlS}$, respectively. Since $N_{LytE}C_{LytF}$ and $N_{LytE}C_{CwlS}$ exhibited cell wall-degrading activity, it was assumed that the C-terminal D,L-endopeptidase domains of $N_{CwlO}C_{LytF}$ and $N_{CwlO}C_{CwlS}$ would exhibit enzyme activity as well; however, it is possible that the N-terminal region of CwlO interfered with the C-terminal D,L-endopeptidase domain activity in $N_{CwlO}C_{LytF}$ and $N_{CwlO}C_{CwlS}$. Next, the subcellular localization of these domain-swapped D,L-endopeptidases was visualized by IFM (Fig. 4). The chimeric proteins containing the LytE N-terminal domain ($N_{LytE}C_{LytF}$ and $N_{LytE}C_{CwlS}$) localized to the cell septa, poles, and lateral cell wall, similar to the localization of LytE-6×FLAG and CWB_{LytE}-6×FLAG. However, $N_{LytF}C_{LytE}$ localized only to the cell septa and poles, like LytF-6×FLAG and CWB_{LytF}-6×FLAG. Only weak fluorescence of the chimeric enzymes containing the N-terminal domain of CwlO ($N_{CwlO}C_{LytF}$ and $N_{CwlO}C_{CwlS}$) was detected. However, enhancing the signal intensity of IFM images revealed that these chimeric enzymes were localized to the sidewall, similar to full-length CwlO and its N-terminal domain. These results demonstrate that the N-terminal domains of D,L-endopeptidases determine their subcellular localization. Finally, we assessed whether these domain-swapped D,L-endopeptidases were able to suppress the *lytE cwlO* synthetic lethality (Fig. 4). The transcription of *cwlO* was induced by xylose in strains expressing LytE or LytF N-terminal domain-containing chimeric enzymes ($N_{LytE}C_{LytF}$, $N_{LytE}C_{CwlS}$, or $N_{LytF}C_{LytE}$), whereas *lytE* gene transcription was induced by IPTG in strains expressing the CwlO N-terminal domain-containing chimeric enzymes ($N_{CwlO}C_{LytF}$ and $N_{CwlO}C_{CwlS}$). After exposure to the appropriate inducer, an aliquot of each culture was washed to remove the inducer, and the cells were inoculated into fresh medium with or without the inducer. OH019 (*lytE*::$N_{LytE}C_{LytF}$ P_{xyl}-*cwlO*) and OH020 (*lytE*::$N_{LytE}C_{CwlS}$ P_{xyl}-*cwlO*) were found to partially suppress the *lytE cwlO* synthetic lethality without xylose induction of *cwlO*. As described above, these chimeric proteins were enzymatically active and detected at the cell septa, poles, and sidewall. However, strains expressing chimeric proteins containing the CwlO N-terminal domain (OH023 [*cwlO*::$N_{CwlO}C_{LytF}$ P_{spac}-*lytE*] and OH024 [*cwlO*::$N_{CwlO}C_{CwlS}$ P_{spac}-*lytE*]), which were not enzymatically active, were localized at the lateral cell wall but not able to grow without IPTG induction of *lytE*. Furthermore, lack of xylose caused the growth arrest of OH022 (*lytE*::$N_{LytF}C_{LytE}$ P_{xyl}-*cwlO*). This strain expressed $N_{LytF}C_{LytE}$, which retained enzymatic activity but was not localized at the cellular sidewall.

Taken together, our findings show that only strains expressing at least one active D,L-endopeptidase localized at the lateral cell wall were able to proliferate. Therefore, we conclude that localization of D,L-endopeptidase activity at the lateral cell wall is essential for cell proliferation.

DISCUSSION

Peptidoglycan forms a network on the outer surface of bacterial cells. The dynamic structure of the peptidoglycan sacculus allows cell growth; therefore, maintaining the balance of peptidoglycan synthesis and disassembly is important. To the best of our knowl-

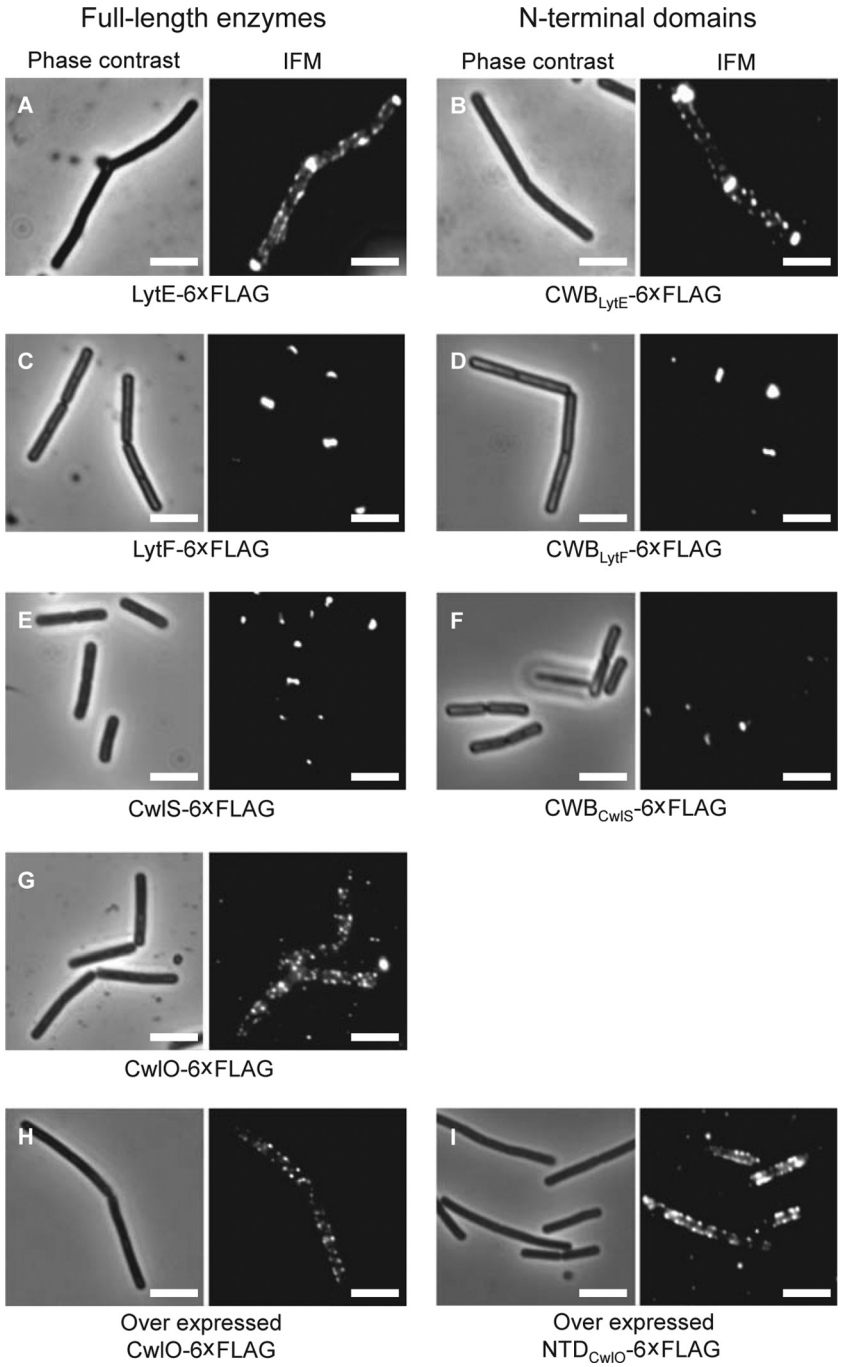

FIG 2 Subcellular localization of full-length D,L-endopeptidases and their N-terminal domains. Phase-contrast and immunofluorescence microscopy analysis of FLAG-tagged proteins. The OD_{600} values at the sampling times were 0.1 for LytE and CwlO and their N-terminal domains (CWB_{LytE} and NTD_{CwlO}, respectively), 0.6 for LytF and its N-terminal domain (CWB_{LytF}), and 2.0 for CwlS and its N-terminal domain (CWB_{CwlS}). (A) WECLytE6FL (LytE-6×FLAG); (B) OH015 (CWB_{LytE}-6×FLAG); (C) WECLytF6FL (LytF-6×FLAG); (D) OH014 (CWB_{LytF}-6×FLAG); (E) WECS6FL (CwlS-6×FLAG); (F) OH016 (CWB_{CwlS}-6×FLAG); (G) WECO6FL (CwlO-6×FLAG); (H) OH013 (overexpressed CwlO-6×FLAG); and (I) OH018 (overexpressed NTD_{CwlO}-6×FLAG). Bars = 5 μm.

edge, the synthetic lethality of *lytE cwlO* in *B. subtilis* is the only report of an autolysin mutant with a serious growth defect (4). In this study, we found that subcellular localization of these enzymes is determined by their N-terminal domains and that synthetic lethality is caused by the lack of D,L-endopeptidase activity at the lateral cell wall. The D,L-endopeptidases required for cell separation (LytE, LytF, and CwlS) were detected at the septa and poles, and the enzymes involved in cell elongation (LytE and CwlO) were detected at the cylindrical part of the cell. These results strongly suggest that the function of these autolysins depends on their subcellular localization. Our findings are consistent with a previous study reporting that a *lytF cwlO* double mutant and a *lytE lytF cwlS* triple mutant were not defective in cell growth (10, 27).

LytE and CwlO may participate in loosening the peptidoglycan

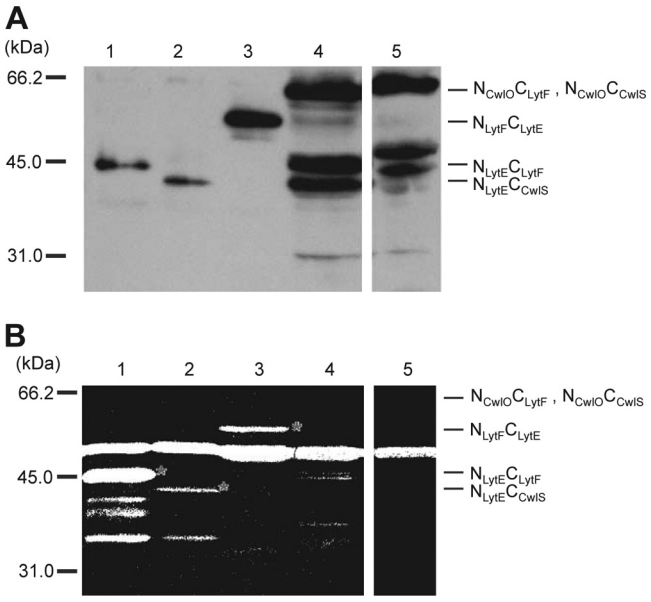

FIG 3 Expression and activity of domain-swapped D,L-endopeptidases. Strains were exposed to 1% xylose or 1 mM IPTG for 2 h to induce P_{xyl}-*cwlO* and P_{spac}-*lytE* expression, respectively. Lanes: 1, OH019 ($N_{LytE}C_{LytF}$ P_{xyl}-*cwlO*, 41 kDa); 2, OH020 ($N_{LytE}C_{CwlS}$ P_{xyl}-*cwlO*, 40 kDa); 3, OH022 ($N_{LytF}C_{LytE}$ P_{xyl}-*cwlO*, 53 kDa); 4, OH023 ($N_{CwlO}C_{LytF}$ P_{spac}-*lytE*, 55 kDa); and 5, OH024 ($N_{CwlO}C_{CwlS}$ P_{spac}-*lytE*, 56 kDa). (A) Domain-swapped D,L-endopeptidases were evaluated by Western blot analysis with an anti-FLAG antibody. Degraded products of the chimeric enzymes appear in lanes 4 and 5. (B) Zymography of the chimeric enzymes using *B. subtilis* cell wall as a substrate. Asterisks indicate clear zones produced by the chimeric enzymes.

sacculus of *B. subtilis* during growth. The cell wall of *B. subtilis* is comprised of multilayered thick peptidoglycan. Electron microscopy images show that the thick peptidoglycan consists of three distinct parts (18). Results of pulse-labeling studies revealed a delay between the incorporation of new material into the cell wall and its eventual appearance in the culture (12, 20). These results suggest that the inner zone of the thick peptidoglycan contains the newly synthesized layers and that the outer zone consists of old peptidoglycan (i.e., inside-to-outside peptidoglycan sacculus formation) (12, 18, 20). Peptidoglycan-synthesizing enzymes are anchored to cytoskeleton proteins (MreB homologs and FtsZ) and localize to the outside surface of the cytoplasmic membrane (6). Thus, the peptidoglycan-synthesizing enzymes are accessible to the inner zone of peptidoglycan. Degradation of the outer zone loosens the cell wall, enabling construction of a new peptidoglycan layer inside the preexisting peptidoglycan sacculus (22). Since *lytE cwlO* double disruption leads to synthetic lethality and impaired cell elongation, these autolysins are strong candidates for participation in the peptidoglycan dynamics. Consistent with this hypothesis, our results show that the cell elongation defect due to the *lytE cwlO* disruption is caused by the absence of D,L-endopeptidase activity at the lateral cell wall. However, results of a pulse-labeling experiment show that the rate of *N*-acetylglucosamine incorporation is not the same for *lytE* and *cwlO* mutants, demonstrating that LytE behavior differs from that of CwlO (4). LytE and CwlO differ in their subcellular localizations and specific activities (28). In addition, CwlO was rapidly degraded and released into culture medium, whereas most of LytE adsorbed to cell surface (27). Taken together, these findings demonstrate that although these two enzymes possess similar D,L-endopeptidase domains, they appear to have different functions in cell growth.

A previous study reported that LytE-3×FLAG transcribed from the *lytE* original promoter was observed at the septa and poles (29). However, slightly overexpressed LytE fused to a green fluorescent protein localized in a helical manner along the cylindrical wall of growing cells in addition to the poles and septa (7). In the present study, we observed the localization of 6 ×FLAG-tagged LytE transcribed from the original *lytE* promoter by IFM (Fig. 2A). The fluorescence intensity of the 6×FLAG fusion protein is greater than that of the 3×FLAG fusion protein, which may be the reason we were able to detect LytE-6×FLAG at the sidewall. The work of Carballido-Lopez et al. (7) strongly suggests that LytE-GFP is localized at the sidewall in a helical manner, similar to the localization pattern of MreB homologs. CwlO-6×FLAG also localized to the lateral cell wall but was not detected at the cell poles or septa (Fig. 2G). Although the fluorescence of the 6×FLAG-tagged CwlO was weak, staggered spots around the sidewall suggested a helical localization pattern. We then investigated whether MreB homologs are involved in the lateral localization of CwlO; however, the mutation of MreB homologs did not alter CwlO localization (data not shown).

Subcellular localization of the N-terminal domains of the four D,L-endopeptidases was similar to that of the corresponding full-length protein, suggesting that localization was determined by their N-terminal domains. This finding was supported by the localization of chimeric enzymes, which was similar to that of their N-terminal domains. The localization of the LytF N-terminal domain at the cell poles and septa was previously reported (30). As expected, the localization of LytE and CwlS was dependent on their N-terminal domains, which contained LysM repeats like that of LytF. Yamamoto et al. (30) also reported a helical localization of LytF-6×FLAG at the sidewall after partial removal of wall teichoic acid, suggesting that the cylindrical localization of N-terminal domains of LytE and CwlS are regulated by wall teichoic acid. Carballido-Lopez et al. (7) reported that LytE localization at the sidewall is dependent on MreBH, indicating that MreBH may regulate wall teichoic acid localization. It was reported that the helical localization of the major wall teichoic acid synthesis proteins was not altered in three *mreB* homolog single mutants (9). However, we note that these cells were cultured with 20 mM $MgCl_2$, which suppresses *mreB* homolog deficiency (14).

The CwlO N terminus contains a COG3883 domain, which is an uncharacterized conserved domain in bacteria. According to Teng et al. (24), a secreted antigen (SagA) from *Enterococcus faecium* containing a COG3883 domain showed broad-spectrum binding to extracellular matrix proteins such as fibrinogen, collagen type I, collagen type IV, fibronectin, and laminin. However, full-length CwlO and its N-terminal domain did not bind some of the matrix proteins evaluated in this study (data not shown). The SagA protein migrated more slowly on cell wall-containing PAGE than on SDS-PAGE, suggesting an interaction between SagA and the cell wall (24); however, the purified CwlO protein did not bind to the cell wall *in vitro* (27). In the present study, we demonstrated the involvement of the CwlO N-terminal domain in cell surface localization. Taken together, these results suggest that CwlO interacts directly, but weakly, with the cell wall or a cell surface protein.

In this study, we found that the subcellular localization of LytE, LytF, CwlS, and CwlO is dependent on their N-terminal domains

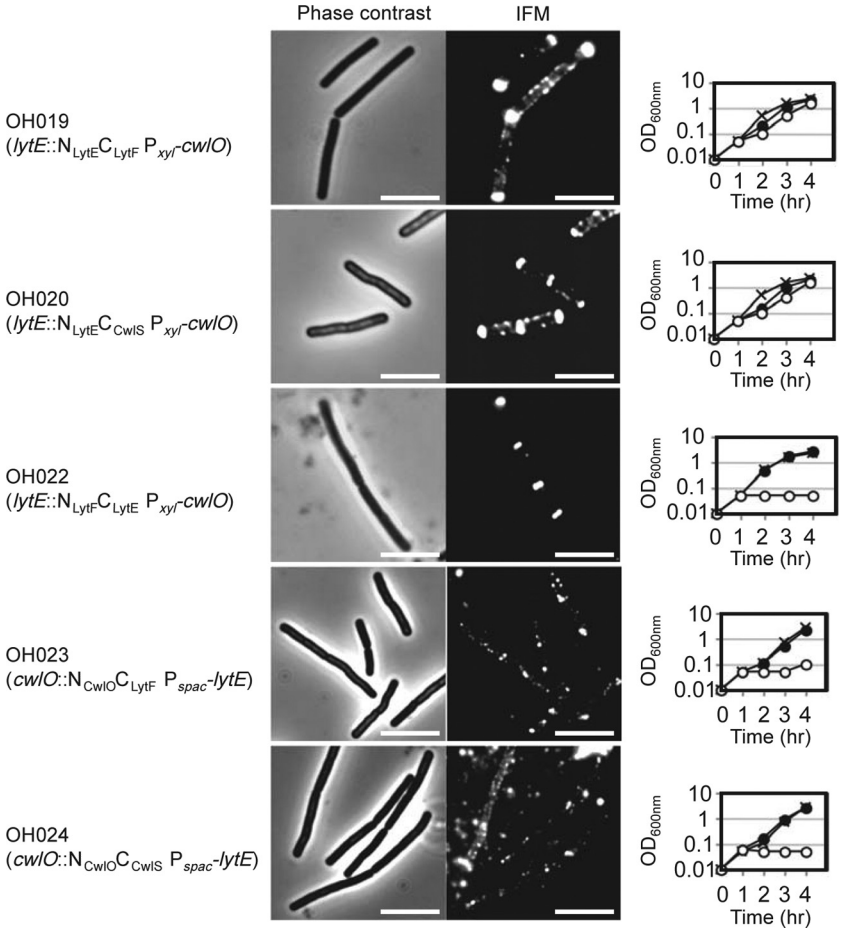

FIG 4 Subcellular localization of domain-swapped D,L-endopeptidases and suppression of the *lytE cwlO* synthetic lethality by these proteins. For microscopic imaging, OH019 (*lytE*::$N_{LytE}C_{LytF}$ P_{xyl}-*cwlO*), OH020 (*lytE*::$N_{LytE}C_{CwlS}$ P_{xyl}-*cwlO*), and OH022 (*lytE*::$N_{LytF}C_{LytE}$ P_{xyl}-*cwlO*) were cultured with 1% xylose to induce CwlO, and OH023 (*cwlO*::$N_{CwlO}C_{LytF}$ P_{spac}-*lytE*) and OH024 (*cwlO*::$N_{CwlO}C_{CwlS}$ P_{spac}-*lytE*) were cultured with 1 mM IPTG to induce LytE. For suppression assays, the strains were grown under the same conditions as those described in Fig. 1. They were cultured with xylose (closed circles) or without xylose (open circles) for P_{xyl}-*cwlO* and IPTG for P_{spac}-*lytE*. The × symbol indicates the wild-type 168 strain. Bars = 5 μm.

and that D,L-endopeptidase activity at the lateral cell wall is essential for cell proliferation. These results strongly suggest that LytE and CwlO are involved in cell elongation and support the inside-to-outside model for peptidoglycan sacculus formation. A more detailed study is necessary to clarify the role of D,L-endopeptidases in peptidoglycan dynamics and to characterize the localization mechanisms of these proteins.

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